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Activity of Novel Adenine Nucleotide Derivatives as Agonists and Antagonists at Recombinant Rat P2X Receptors

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Abstract

Strategy, Management and Health Policy

Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV
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The effects of structural modifications of adenine nucleotides previously shown to enhance either agonist (2-thioether groups) or antagonist (additional phosphate moieties at the 3'- or 2'-position) properties at P2Y₁ receptors were examined at recombinant rat P2X₁, P2X₂, P2X₃, and P2X₄ receptors expressed in *Xenopus* oocytes. The potency of P2Y₁ agonists HT-AMP (2-(hexylthio)adenosine-5'-monophosphate) and PAPET (2-[2-(4-aminophenyl)ethylthio]adenosine-5'-triphosphate) was examined at P2X receptors. Both nucleotides showed a preference for the Group I (α,β-meATP-sensitive, fast-inactivating) P2X sub-units. HT-AMP was 5-fold more potent than ATP at P2X₃ receptors and a partial agonist at all except P2X₂ receptors, at which it was a full agonist. The efficacy of HT-AMP was as low as 23% at P2X₄ receptors. PAPET was a weak partial agonist at rat P2X₄ receptors and a nearly full agonist at the other subtypes. At rat P2X₃ receptors, PAPET was more potent than any other known agonist (EC₅₀ = 17 ± 3 nM). MRS 2179 (N⁶-methyl-2'-deoxyadenosine 3', 5-bisphosphate, a potent P2Y₁ receptor antagonist) inhibited ATP-evoked responses at rat P2X₁ receptors with an IC₅₀ value of 1.15 ± 0.21 μM. MRS 2179 was a weak antagonist at rat P2X₃ receptors, with an IC₅₀ value of 12.9 ± 0.1 μM, and was inactive at rat P2X₂ and P2X₄ receptors. Thus, MRS 2179 was 11-fold and 130-fold selective for P2Y₁ receptors vs. P2X₁ and P2X₃ receptors, respectively. MRS 2209, the corresponding 3'-deoxy-2'-phosphate isomer, was inactive at rat P2X₁ receptors, thus demonstrating its greater selectivity as a P2Y₁ receptor antagonist. Various adenine bisphosphates in the family of MRS 2179 containing modifications of either the adenine (P2Y₁ antagonists with 2- and 6-substitutions), the phosphate (a 3',5'-cyclic diphosphate, inactive at P2Y₁ receptors), or the ribose moieties (antagonist carbocyclic analogue), were inactive at both rat P2X₁ and P2X₃ receptors. An anhydrohexitol derivative (MRS 2269) and an acyclic

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derivative (MRS 2286), proved to be selective antagonists at P2Y₁ receptors, since they were inactive as agonist or antagonist at P2X₁ and P2X₃ receptors.

Keywords

ion channel; oocytes; purines; ATP derivatives; bisphosphates; deoxyadenosine derivatives

INTRODUCTION

Extracellular ATP and other adenine nucleotides play an important physiological role in the central and peripheral nervous systems and in the immune, cardiovascular, renal, and musculo-skeletal systems through the activation of P2 receptors [North and Barnard, 1997; Burnstock, 1996]. Two families of P2 receptors have been defined [Abbracchio and Burnstock, 1994]: ligand-gated cation channels (P2X subtype), activated by adenine nucleotides, and G-protein coupled receptors (P2Y subtype), most of which are activated by both adenine and uracil nucleotides. Several of the P2Y subtypes are activated exclusively by uracil nucleotides [Communi and Boeynaems, 1997]. P2X₁₋₇ and P2Y_{1,2,4,6,11} designations have been unambiguously assigned to mammalian nucleotide receptors [Burnstock and King, 1996; Fredholm et al., 1997; Communi et al., 1997], although there is still uncertainty about the correspondence of these cloned sequences to the pharmacological phenotypes of native P2 receptors. The P2X₃ receptors found in dorsal root ganglion neurons are a therapeutic target for pain control [Burgard et al., 1999].

Synthetic ligands which display high potency and/or selectivity at various subtypes of P2 receptors are currently being designed [Lambrecht et al., 1996; Williams and Bhagwat, 1996; Jacobson et al., 1997; Fischer, 1999]. One of the first advances in the design of highly potent P2 receptor agonists was the introduction of 2-thioether groups, i.e., analogs of 2-MeSATP. For example, PAPET (2-[2-(4-aminophenyl)ethylthio]adenosine-5'-triphosphate), **1a**, and 2-cyanoethylthioATP, **1b** (Fig. 1) were shown to activate turkey erythrocyte P2Y₁ receptors with EC₅₀ values of 2.5 and 1.0 nM, respectively [Fischer et al., 1993]. The linkage of ATP with bulky 2-thioether groups not only enhanced affinity, but also decreased susceptibility to degradation by nucleotidases [Zimmer et al., 1993]. In the series of 5'-monophosphates, certain 2-thioether groups enhanced potency as P2 receptor agonists. Thus, Boyer et al. [1996] found that HT-AMP (2-(hexylthio)adenosine-5'-monophosphate), **2** (Fig. 1) was 72-fold more potent than ATP in activating turkey P2Y₁ receptors, with an EC₅₀ of 59 nM. Compound **3** (2-(5-hexenyl)thioadenosine-5'-triphosphate, MRS 2055), is a moderately potent agonist at the adenylate cyclase-coupled P2Y receptor [P2Y_{AC}; Boyer et al., 1996] and inactive at P2Y₁ receptors.

Selective antagonists have been designed for P2Y₁ receptors [Camaioni et al., 1998; Boyer et al., 1998; Nandanan et al., 1999] and the yet uncloned P2T receptors [Ingall et al., 1999]. MRS 2179 (N⁶-methyl-2'-deoxyadenosine 3',5'-bisphosphate), **4**, a bisphosphate derivative (Fig. 1), was found to be a pure, competitive antagonist at turkey and human P2Y₁ receptors, with a K_B value of 100 nM [Boyer et al., 1998]. Before such ligands can be used as definitive pharmacological probes, for example, in platelets, in which P2X₁, P2Y₁, and P2Y_{AC} receptors coexist [Jin et al., 1998; Hechler et al., 1998; Fagura et al., 1998], it is necessary to explore their activity at the full range of P2 receptors [Bianchi et al., 1999]. This is the first study of the activity of these novel agents at recombinant P2X receptors.

MATERIALS AND METHODS

Adenosine nucleotide derivatives were synthesized as reported [Fischer et al., 1993; Boyer et al., 1996; Nandanan et al., 1999; Kim et al., 2000].

Antagonist Activity at Recombinant P2X Receptors

Xenopus oocytes were harvested and prepared as previously described [King et al., 1997]. Defolliculated oocytes were injected cytosolically with 40 nl of a solution of cRNA of rat P2X₁, P2X₃, or P2X₄ receptors (1 µg/ml) or rat P2X₂ receptors (0.002 µg/ml) incubated for 24 h at 18°C in Barth's solution and kept for up to 12 days at 4°C until used in electrophysiological experiments.

ATP-activated membrane currents ($V_h = -90$ mV) were recorded from cRNA-injected oocytes using the twin-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1–2 MΩ tip resistance) and current-recording microelectrodes (5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 ml/min, at 18°C) containing (in mM): NaCl, 110; KCl, 2.5; HEPES, 5; BaCl₂, 1.8, adjusted to pH 7.5.

ATP was superfused over oocytes for 120 sec then washed out for a period of 20 min. The agonist concentration (1 µM for P2X₁, 30 µM for P2X₂, 3 µM for P2X₃, or 10 µM for P2X₄) was approximately equal to the EC₇₀ value at each subtype. For inhibition curves, data were normalized to the current evoked by ATP at pH 7.5. Test substances were added for 5 min prior to ATP exposure; all compounds were tested for reversibility of their effects. The concentration required to inhibit the ATP-response by 50% (IC₅₀) was taken from Hill plots constructed using the formula $\log(I/I_{\max} - I)$, where I was the current evoked by ATP in the presence of an antagonist. Data are presented as mean ± SEM (n = 4) for data from different batches of oocytes.

RESULTS

ATP agonists were tested in functional assays of recombinant rat P2X_{1,2,3,4} receptors expressed in *Xenopus* oocytes (Fig. 2). Values for potency (EC₅₀) in activating membrane currents and maximum efficacy (as percent) are reported (Table 1). HT-AMP was 5-fold more potent than ATP at P2X₃ receptors and a partial agonist at all except P2X₂ receptors, at which it was a full agonist. The efficacy of HT-AMP was as low as 23% at P2X₄ receptors. PAPET was a weak partial agonist at rat P2X₄ receptors and a nearly full agonist at the other subtypes. At rat P2X₃ receptors, PAPET was more potent than any other known agonist (EC₅₀ = 17 ± 3 nM).

Various adenine bisphosphates (Fig. 3) in the family of MRS 2179 (a potent P2Y₁ receptor antagonist) containing modifications of either the adenine (2- and 6-substitutions), the phosphate (a 3',5'-cyclic diphosphate), or the ribose moieties (carbocyclic and anhydrohexitol analogs), were examined for the ability to interact with P2X receptors. MRS 2179 (Fig. 1), itself, inhibited ATP-evoked responses at rat P2X₁ receptors with an IC₅₀ value of 1.15 ± 0.21 µM (Fig. 4a). MRS 2179 was a weak antagonist at rat P2X₃ receptors, with ~40% inhibition occurring at 10 µM, and was inactive at rat P2X₂ and P2X₄ receptors (Fig. 4b). The IC₅₀ value of MRS 2179 at rat P2X₃ receptors was determined to be 12.9 ± 0.1 µM. At rat P2X_{1,2,3,4} receptors, MRS 2179 displayed no agonist properties in the absence of ATP.

Figure 3 shows the structures of a variety of bisphosphate derivatives that have been tested at turkey erythrocyte P2Y₁ receptors as antagonists of the effects of 2-MeSADP. We have

currently tested these compounds at recombinant rat P2X₁ and P2X₃ receptors. All of the bisphosphate analogs shown in Figure 3, i.e., compounds **5–10**, were demonstrated to be inactive at P2X₁ and P2X₃ receptors, either as agonists (in the absence of ATP) or antagonists (at 1 or 10 μ M for rat P2X₁ and at 1 μ M for rat P2X₃ receptors, vs. either 1 or 3 μ M ATP, respectively).

Compounds **5a–5c**, which are 2-substituted analogs of MRS 2179, and MRS 2209, **6**, the 3'-deoxy-2'-phosphate isomer of MRS 2179, were inactive at rat P2X₁ and P2X₃ receptors, thus demonstrating their selectivity as P2Y₁ receptor antagonists. Similarly, other ribose-modified bisphosphate antagonist of P2Y₁ receptors, in which the ribose ring was replaced with either cyclopentane (**7**) or anhydrohexitol (**9**), were found to be inactive at both rat P2X₁ and P2X₃ receptors. Compounds **7**, a cyclic diphosphate corresponding to an anhydride of MRS 2179, and **10**, a six-membered ring morpholine derivative having an ethyl phosphonate on a ring nitrogen, were inactive at P2Y₁ receptors as either agonists or antagonists and were found to be similarly inactive at P2X₁ and P2X₃ receptors. The acyclic nucleotide analog MRS 2286 [Kim et al., 2000], **11**, is an antagonist at turkey P2Y₁ receptors, but is inactive at both rat P2X₁ and P2X₃ receptors.

DISCUSSION

Since agonist potency at P2X₁ and P2X₃ receptors is often linked, an aim of this study has been to distinguish these two subtypes with selective ligands. At rat P2X₃ receptors, PAPET, **1**, was more potent than any other known agonist, with an EC₅₀ value of 17 nM. This derivative was a full agonist at P2X₁, P2X₂, and P2X₃ receptors and a partial agonist at P2X₄ receptors. The mono-phosphate derivative HT-AMP, **2**, appeared to be a partial agonist at P2X₁, P2X₃, and P2X₄ receptors, and a weak full agonist at P2X₂ receptors. Both of these 2-alkylthio nucleotide derivatives showed a preference for the Group I (α , β -meATP-sensitive, fast-inactivating) P2X subunits, i.e., P2X₁ and P2X₃ receptors. Thus, PAPET was 600- and 900-fold selective for P2X₃ vs. P2X₂ and P2X₄ receptors, respectively, with nanomolar potency. HT-AMP was 510- and 58-fold selective for P2X₃ vs. P2X₂ and P2X₄ receptors, respectively. The preference for Group I was much more pronounced for the 2-thioether derivatives, including 2-MeSATP [King, 1998], than for ATP itself. 2-MeSATP was reported to have the following EC₅₀ values (in μ M): 0.4 (P2X₁), 7.1 (P2X₂), 0.2 (P2X₃), and 74 (P2X₄) [King, 1998]. Thus, PAPET appears to be more potent than 2-MeSATP at recombinant P2X₁ and P2X₃ receptors expressed in oocytes. The utility of PAPET and HT-AMP and similar 2-thioethers derivatives as potent P2 agonists *in vivo* may be further enhanced due to increased resistance to nucleotidases [Zimmet et al., 1993].

Among bisphosphate derivatives tested, only MRS 2179, **4**, showed any antagonist properties at P2X receptors. Thus, MRS 2179 remained a moderately selective P2Y₁ receptor antagonist, with ratios of selectivity vs. P2X₁ and P2X₃ receptors of 11- and 130-fold, respectively, and, as reported, is inactive at P2Y₂, P2Y₄, and P2Y₆ receptors [Boyer et al., 1998]. MRS 2179 has also been found to be inactive at P2Y_{AC} receptors in platelets [Boyer et al., 1998] and at P2Y₁₁ receptors (B. Torres, A. Zambon, and P. Insel, unpublished observations), as well as P2X₂ and P2X₄ receptors. MRS 2179 (100 μ M) is also inactive as either substrate or inhibitor of recombinant rat ecto-ATPase and ecto-apyrase expressed in CHO cells (C. Hoffmann, K. Jacobson, and H. Zimmermann, unpublished observations).

Nucleotides that distinguish between P2X₁ and P2Y₁ receptors may be useful pharmacological probes to study aggregation effects in platelets. Among the most important findings of the present study are that compounds **5–7**, **9**, and **11**, which bind to P2Y₁ receptors in the micromolar range, appear to be very selective for that subtype. The

substitution of MRS 2179 at the 2-position with a chloro-, methylthio-, or amino- group, **5a–5c**, abolished activity at P2X_{1–4} receptors, and similarly, the 2', 5'-isomer of MRS 2179, **6**, was inactive at P2X receptors. The carbocyclic derivative, **7**, was a moderately potent P2Y₁ receptor antagonist (IC₅₀ 2.53 μ M) and inactive at P2X_{1,3} receptors. Thus, compounds **5a–5c**, **6**, and **7**, which are relatively potent as antagonists at P2Y₁ receptors (IC₅₀ < 3 μ M), were highly selective at that subtype.

The anhydrohexitol derivative **9**, an antagonist at P2Y₁ receptors, appeared to be a selective antagonist of P2Y₁ receptors, i.e., to the extent that it has no effect at P2X_{1,3} receptors. The corresponding 6-NH₂ derivative (MRS 2255), a pure agonist at P2Y₁ receptors with an EC₅₀ value of 2.99 μ M [Nandanan et al., 1999], for stimulation of phospholipase C in turkey erythrocytes, should also be evaluated at P2X receptors and may prove to be a selective P2Y₁ agonist. A selective P2Y₁ receptor agonist may be useful as a hypotensive agent or in the treatment of diabetes, while a selective P2Y₁ receptor antagonist may be useful as an antithrombotic agent.

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Abbreviations

ATP	adenosine 5'-triphosphate
EC₅₀	50% effective concentration
EFS	electrical field stimulation
HT-AMP	2-hexylthioadenosine-5'-monophosphate
IC₅₀	50% inhibitory concentration
K_B	Schild constant
me-ATP	adenosine-5'-methylenetriphosphate, (α,β) or (β,γ) isomers
2-MeSATP	2-methylthioadenosine-5'-triphosphate
MRS 2055	2-(5-hexenyl)thioadenosine-5'-triphosphate
MRS 2179	N ⁶ -methyl-2'-deoxyadenosine 3', 5-bisphosphate
MRS 2209	N ⁶ -methyl-3'-deoxyadenosine 2', 5-bisphosphate
PAPET	2-[2-(p-aminophenyl)ethylthio]adenosine-5'-triphosphate
PPADS	pyridoxal-5'-phosphate-6-phenylazo-2,4-disulfonate
SAR	structure–activity relationship

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Fig. 1.
Structure of adenosine-5'-triphosphate and monophosphate 2-thioether derivatives previously found to be potent P2 receptor agonists.

Fig. 2. Effects of various analogs ($n = 4$) as indicated on current induced at recombinant rat P2X receptors, expressed in *Xenopus* oocytes. The twin electrode voltage clamping technique was used. The medium consisted of Ba^{2+} Ringer's buffer at pH 7.50.

Figure 3.

Structures of deoxyadenosine bisphosphate derivatives previously tested as P2Y₁ receptor agonists/antagonists [Camaioni et al., 1998; Nandanan et al., 1999; Kim et al., 2000]. IC₅₀ values (μM) for inhibition of phospholipase C elicited by 10 nM 2-MeSADP at the turkey erythrocyte P2Y₁ receptors were found to be: **5a**, 0.206; **5b**, 0.362; **5c**, 1.85; **6**, 0.324; **7**, 2.53; **8**, >50; **9**, 1.64; **10**, no effect; **11**, 0.84.

Fig. 4. Effects of MRS 2179 on current induced by activation of recombinant rat P2X₁ and P2X₃ receptors (concentration–response curve, **a**) and rat P2X_{1–4} receptors (at 10 μM, **b**), expressed in *Xenopus* oocytes (n = 8). The IC₅₀ at P2X₁ receptors was found to be 1.15 ± 0.21 μM. The twin electrode voltage clamping technique was used. The medium consisted of Ba²⁺ Ringer's buffer at pH 7.50.

Table 1

Agonist Potency and Efficacy at Rat P2X 1,2,3,4 Receptors

Receptor	ATP	PAPET	HT-AMP
EC ₅₀ Values (μM)			
P2X ₁	0.30 ± 0.01	0.098 ± 0.010	0.84 ± 0.11
P2X ₂	11.0 ± 3.2	10.2 ± 1.0	180 ± 60 ^a
P2X ₃	1.85 ± 0.37	0.017 ± 0.003	0.35 ± 0.22
P2X ₄	4.14 ± 1.34	15.3 ± 5.3	20.4 ± 0.31
Efficacy (maximum) receptor			
P2X ₁	100%	91 ± 5%	49 ± 2%
P2X ₂	100%	91 ± 2%	100% ^b
P2X ₃	100%	83 ± 2%	50 ± 2%
P2X ₄	100%	27 ± 2%	23 ± 1%

^aEstimated: the log concentration–response curve for HT-AMP at P2X₂ receptors did not reach a definable maximum count and accordingly, the stated EC₅₀ value is an estimate.

^bThe maximum was not determined over the concentration range studied.